IV. AGAR ANAPHYLATOXIN: RAT SERUM

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SYNOPSIS

The early work on trypanosomes demonstrated that rat serum gives rise to a very active anaphylatoxin, and consequently, in the major part of the agar studies this reagent was used. There is no evidence to show that rat serum has been used for this purpose by other investigators and yet, as will be shown, it is incomparably the best,* since it can be toxified in a few minutes to such an extent that 0.25 c.c. will cause typical acute fatal shock. The speed and ease of the poison-production are remarkable when it is borne in mind that the primary toxicity of normal rat serum is no greater than that of the guinea-pig or rabbit.

In the beginning, considerable variation in results was encountered, just as in the work with guinea-pig and rabbit serum. It was reasonably clear, however, that this diversity was chiefly due to imperfect conditions as regards distribution of the agar in the serum, and for that reason much time and endeavor were devoted to developing the best methods of work. But even with improved methods it was found that occasional variation was encountered, which served to show that the rat serum was not always uniform; a fact which corresponds with the observations made in regard to the sera of the guinea-pig and rabbit.

The rat blood was drawn from the heart and defibrinated in the tube pipet by means of the glass rod, the technic being the same as that given in Part I. As a rule, pooled fresh sera were employed, but equally good results were

* The serum of the musk-rat (Castor zibethicus) is probably as toxifiable as that of the rat. In one experiment with sol-gel Mixture 6, after incubation at 37 C. for 30 minutes, the serum in dose of 0.5 c.c. gave a typical fatal shock in 4 minutes 45 seconds in a guinea-pig weighing 173 gm.; 0.25 c.c. gave a severe shock.

obtained with sera which had been iced or kept at room temperature for 24 hours. The presence of lipoids did not seem to interfere with the poison-production. Serum prepared in this way is ordinarily not harmful, in dose of from 5 to 6 c.c. given intravenously, to 200-gm. guinea-pigs. But, after incubation at 37 C. for some minutes, it may produce an acutely fatal anaphylactic shock in dose of 4 c.c. (Part VIII). It has even at times been found to produce the same effect in dose of 1 c.c., when the incubation had extended over a long period of time (20 to 120 hours). In this autotoxification, the anaphylatoxin is produced without the known intervention of any foreign agent. This fact indicates an inherent lability in the serum which is merely accentuated or accelerated by the presence of alien substances, such as agar, trypanosomes, distilled water, etc.

The preparation of the agar solution has already been described. As pointed out, it can be sterilized at 100, 110, 120, 130, or 140 C. without affecting the toxifying power. Further evidence of this will be presented in Table 47.

EXPERIMENTS WITH AGAR SOL, 37 C.

At the outset, solid or semisolid gel was used, but frequent irregularities and failures seemed to indicate that the trouble was due largely to inability to duplicate a given state of division. Accordingly, it was hoped to overcome this difficulty by the use of a hydrosol. For this purpose, the agar after being liquefied in the water bath at 100 C., was transferred to the Roux bath at 37 C. for from 2 to 5 hours. The desired amount of sol was then added to the serum, which previously had been warmed to 37 C.

A comparison of the results of these early tests (Table 46) with those obtained by the sol-gel method (Table 49) will show the superiority of the latter procedure, in which the 0.25-c.c. lethal dose was attained in 5 mixtures after an incubation of only 15 minutes. Table 46 is a composite of 36 separate experiments, in each of which the 37 C. sol was added to 2 c.c. of rat serum. The mixture was then shaken, sometimes very gently and at other times very vigorously, after which it was incubated for the stated time. It was then centrifugated at 3000 revolutions for 5 minutes, and the clear fluid injected. These tests are grouped according to the amount of agar used. The 7 mixtures indicated in the first column correspond with those heretofore used (Table 23), and to the sol-gel mixtures to be discussed later. The ratio is that of the agar solution to the serum.

It will be noted that 0.25 c.c. proved fatal in 2 instances (6a and 7), and then only after prolonged incubation. Tests 4 to 7 were made with the same pooled serum. Apparently, the best mixture is 6*a*, since this on incubation for 7½ minutes became fatal in dose of 0.5 c.c.; when incubated for 15 minutes it was nearly fatal in dose of 0.25 c.c.

The very severe result with mixture 9 is of interest as showing that an excess of agar does not interfere with poison-production. Nathan,[1] working with agar gel and guinea-pig serum, the mixture (0.5 : 1) corresponding with No. 8, did not succeed in producing a fatal anaphylatoxin, tho the amount injected was 4.5 c.c.

TABLE 46

ACTION OF AGAR SOL (37 C.) ON RAT SERUM
(MIXTURES KEPT AT 37 C., THEN CENTRIFUGATED AND TESTED)

Mixture		Guinea-Pig		Serum		Result
No.	Ratio	No.	Weight	B. C.* at 37 C.	c.c. (intravenously)	
1	0.005:1	1	205	15 min.	1.0	Very slight
		2	250	1 hr.	"	Very slight
		3	173	2½ hr.	"	Very severe
		4	170	3 hr.	"	Slight
		5	175	6 hr.	"	3′ 5″
		6	170	22 hr.	0.5	3′30″
		6a	170	22 hr.	0.25	1½ hr.
		7	170	29 hr.	0.25	3′50″
4	0.025:1	8	175	7½ min.	1.0	Very slight
		9	170	15 min.	1.0	3′40″
		10	202	30 min.	0.5	Fair
		11	225	1 hr.	1.0	3′40″
		12	218	1 hr.	1.0	2′50″
5	0.05:1	13	240	7½ min.	0.5	Very slight
		14	240	7½ min.	0.5	Very slight
		15	206	15 min.	0.5	Moderate
		16	227	15 min.	1.0	9 hr.
		17	197	30 min.	1.0	3′40″
		18	220	1 hr.	1.0	Severe
6a	0.125:1	19	220	7½ min.	0.5	3′50″
		20	173	7½ min.	0.5	3′15″
		21	177	15 min.	0.25	Near-kill
		22	180	1 hr.	0.5	3′55″
7	0.25:1	23	225	2½ min.	0.5	Very slight
		24	229	7½ min.	"	Very slight
		25	220	7½ min.	"	Slight
		26	215	7½ min.	"	Very slight
		27	175	15 min.	"	3′30″
		28	180	15 min.	"	3′40″
		29	240	1 hr.	"	6′20″
		30	210	1 hr.	"	Very severe
		31	245	1 hr.	"	Very slight
		32	175	2¼ hr.	"	3′35″
		32a	170	4 hr.	"	4′20″
8	0.5:1	33	220	15 min.	1.0	4
9	1:1	34	196	15 min.	0.6	Near-kill

* B. C. = before centrifugation.

EXPERIMENTS WITH AGAR GEL

It has been pointed out that Bordet allowed the sterilized agar to gel over night, after which it was thoroughly shaken till it became a thick fluid; 1 volume of this softened agar was then added to 5 volumes

[1] Ztschr. f. Immunitätsf., 1913, 17, p. 480.

of serum, and the whole mixed and incubated at 37 C. for 2 hours; whereupon the mixture was centrifugated and the clear serum injected in dose of 4 to 5 c.c. Inasmuch as the previous work with trypanosomes had shown that rat serum could be rendered toxic in dose of 1 c.c. or less, by an incubation of only 15 minutes, it was felt that a like result could be obtained with agar. Accordingly a mixture of 1 part of shaken agar and 4 parts of serum was prepared and tested. As expected, after incubation for 1 hour the treated serum produced the typical fatal shock in dose of 1 c.c. Further tests soon showed that not only could this toxic dose be obtained after incubation for 15 minutes, but likewise the minimal lethal dose of 0.25 c.c. This fact was utilized as the basis of an extensive series of tests having for their object the demonstration of the presence of a ferment (anaphylase). It was soon found that the production of a serum of maximal toxicity was more an accident than a certainty, and this led to a long study of the underlying conditions.

As a rule, no difficulty was experienced in obtaining a toxic serum the lethal dose of which was 1 or even 0.5 c.c. This result was secured equally well after incubation for 7½ or for 15 minutes, and, moreover, a serum gave as good results after 24 hours as when perfectly fresh. It seemed, however, that much could be learned by determining the conditions which favored maximal toxicity.

At the outset it was desirable to ascertain whether the mode of sterilization in any way affected the findings.

For this purpose, a number of agar solutions were prepared by heating the agar with distilled water (0.5 gm. + 100 c.c.) in an autoclave at different temperatures. After sterilization the flasks of agar solution were either kept in the room or were placed in snow or ice to gel. The gel was then broken up by shaking and 1 part of the softened mass was then added to 4 parts of the rat serum and the mixtures vigorously shaken for 1 minute; they were then placed at 37 C. for the desired length of time, after which they were centrifugated at 3000 revolutions for 4 minutes, and the clear serum then injected. The results of these tests are given in Table 47.

With reference to these tests it may be stated that the same pooled serum was employed in the work with the 102, 110, and 120 C. agar; another pool, previously iced for 11 hours, was used with the 130 C. agar; that used with the 140 C. agar had been iced for 23 hours.

While this table would seem to show that the agar sterilized at 130 C. is more active than the others, as a matter of fact such conclusion is not justified, since the results are not constant. Frequently, the 130 C. agar failed to render a serum toxic in dose of 0.25 or even 0.5 c.c. On the other hand, the 120 C. agar seemed to be more certain in its action and for that reason was employed in most of the subsequent work. It may be added that the 110 C. agar, under like conditions, usually rendered serum toxic in dose of 0.5 c.c. in 7½ minutes.

Taken by and large, it is evident that the heat of sterilization, as employed, does not materially affect the inducing power of the agar.

In harmony with all similar observations is the result with Test 13 of Table 47, which shows that mere contact of agar and serum is not sufficient to produce anaphylatoxin. A like mixture placed in cracked ice for 1 hour and then tested was equally negative. Time and a moderate temperature are necessary to develop the reaction. At 37 C. even 2½ minutes (No. 14) are sufficient to toxify 1 c.c.; while 7½ minutes may be quite enough to toxify 0.25 c.c.

TABLE 47

EFFECT OF STERILIZATION UPON THE TOXIFYING POWER OF AGAR GEL

Sterilization of Agar		Guinea-Pig		Serum		Result
C.	min.	No.	Weight	B. C. at 37 C. (min.)	c.c. (intravenously)	
102	15	1	180	7½	0.5	5′20″
		2	198	7½	0.25	3 hr.
		3	182	15	0.5	3′30″
		4	179	15	0.25	Fair shock
110	60	5	205	7½	1.0	3′35″
		6	225	7½	0.5	Fair
		7	235	15	0.5	3′25″
		8	185	15	0.25	Near-kill
120	30	9	178	7½	0.5	4′25″
		10	175	7½	0.25	Slight
		11	203	15	0.5	2′30″
		12	202	15	0.25	Slight
130	30	13	225	0	1.0	Slight
		14	170	2½	1.0	3′5″
		15	225	2½	0.5	Severe
		16	225	7½	0.5	3′50″
		17	170	7½	0.25	3′30″
		18	170	15	0.5	4′10″
		19	178	15	0.25	5′15″
		20	180	15	0.13	Slight
140	30	21	170	7½	0.5	3′10″
		22	172	7½	0.25	3½ hr.
		23	190	15	0.5	3′35″
		24	183	15	0.25	Slight

These facts are of much importance, being in line with many other observations all of which go to show that under favorable conditions anaphylatoxin-production occurs at great speed. The speed of production with agar is the same as that with trypanosomes, both being able to toxify rat serum in 2½ minutes so that it will kill in dose of 1 c.c.

In 2 instances (Nos. 2 and 22), it will be noted that 0.25 c.c. produced a severe shock followed by a subacute death; the initial shock was succeeded by severe prostration, paralysis of the extremities, and a progressive and rapid fall of temperature to 26 C. In general it may

be said that the more severe the shock, the more rapid the recovery. The subacute deaths from the rat anaphylatoxin are most unusual, tho they are common enough with rabbit serum.

As pointed out the results obtained with the 130 C. agar, as given in Table 47, appeared to show that such agar was the most active. Accordingly, this material was used in a large number of experiments, but with most discouraging results. With the utmost care in duplicating conditions, the results varied; the treated sera would sometimes fail to kill in dose of 1 c.c.; often they failed with 0.5 c.c., and it seemed almost impossible to obtain again the 0.25-c.c. lethal dose. In the course of 3 weeks not less than 10 different lots of 130 C. agar were tested. The conclusion was finally reached that apart from variations in the different lots of serum, an essential factor was a fugacious state of the agar gel.

Accordingly, and in order to secure the utmost uniformity, in subsequent experiments the agar was kept always in small Erlenmeyer flasks of 150-c.c. capacity or less. Immediately before use it was liquefied by heating in a water bath at 100 C. for 15 minutes, after which it was placed to gel in cracked ice (0 C.) for 1 hour. Icing for a longer period was found to be undesirable as it resulted in a harder gel that was more difficult to break up in the subsequent operations. The solid agar gel was then measured out directly by means of a pipet, which was cut off at the lowest graduation line, or the gel was first broken up by moderate swinging for 20 seconds. Such agar was then added to the previously iced serum and mixed either by tapping, or by swinging for 1 minute, or by means of a finely drawn-out pipet. The mixture thus prepared was now incubated, centrifugated, and tested.

Adherence to these conditions gave most excellent results, as will be seen from Table 48, in which are presented tests made on 3 consecutive days.

Fourteen different mixtures, each containing 2 c.c. of serum, were employed. For Nos. 1 to 4 the ratio of agar to serum was 0.125:1, while for the others it was 0.25:1. The agar employed for Nos. 13 and 14 was chilled at 0 C.; that for the others was gelled at —2 C., in a freezing mixture consisting of 1 part of salt and 50 parts of cracked ice. For Tests 5 and 9 to 12, the gel was not shaken up but was transferred to the serum as a solid cylinder; for the other tests it was thoroughly shaken for about 20 seconds. After the addition of the agar each mixture was shaken vigorously for 1 minute, except Test 13, which was given 2 minutes. The result of this test compared with that of No. 14, the same serum being used, appears to indicate that a slight change in the manipulation can affect the outcome. It is more likely, however, in view of the results shown in Table 45 that the resistance of the recipient was greater than usual.

Of the 5 different agars used in these experiments, those employed for Tests 9 and 12 were sterilized at 120 C. for 5 minutes, whereas the others were given 130 C. It is to be noted that the mixtures used for

Tests 1 to 4, which contain one-half the amount of agar present in those of Nos. 5 to 8, yield as good, if not better, results, tho the same pooled serum was used for all. It will also be seen that incubation at 37 C. for 7½ minutes may yield a lethal dose of 0.25 c.c. (No. 14), whereas 15 minutes sufficed in all the tests except in No. 13, which has been referred to. In test 10a a very severe shock was produced by as small a dose as 0.13 c.c.

TABLE 48

ACTION OF AGAR GEL (0 C.) ON RAT SERUM (0 C.)
(MIXTURES KEPT AT 37 C., THEN CENTRIFUGATED AND TESTED)

Mixture		Guinea-Pig		Serum		Result
No.	Ratio	No.	Weight	B. C. at 37 C. (min.)	c.c. (intravenously)	
6a	0.125:1	1	180	7½	0.25	Severe
		2	185	15	"	4′ 5″
		3	200	30	"	3′25″
		4	175	60	"	3′40″
7	0.25:1	5	200	15	"	8′30″
		6	170	"	"	3′25″
		7	200	30	0.5	Slight
		8	170	60	0.25	Very slight
		8a	210	"	0.5	2′55″
		9	185	15	0.25	2′50″
		10	170	"	0.15	2′20″
		10a	178	"	0.13	Very severe
		11	185	"	0.25	4′55″
		12	181	"	"	3′20″
		13	182	7½	"	Slight
		14	185	"	"	3′50″

In experiments of this type the results depend very largely on the extent to which the agar is broken up in the process of shaking the mixture. With a moderate shake, many large lumps of agar, 2 mm. or more in diameter, can be seen in the serum. This is especially likely to occur when the gel is used at once after it has set at 0 C. Inasmuch as the fineness of the division cannot be controlled, it follows that the results by this method are not always as favorable as those given in Table 48. The general conviction arrived at after many trials was that the agar should be in the form of a hard gel, and thoroughly broken up while in that state; a transition toward the sol form apparently weakens the reaction. Excessive shaking of the gel before use, or a long standing of such shaken agar, impairs its efficiency.

EXPERIMENTS WITH SOL-GEL AGAR

It has been shown that it is possible to obtain in a few minutes by means of the agar gel a toxic serum such that 0.25 c.c. is acutely fatal. The method, however, did not yield this lethal dose with as much regu-

larity as was desirable, largely because of the difficulty of securing perfect distribution of the agar through the serum.

This difficulty was eventually overcome by devising the sol-gel method (Part II), in which as previously stated, the desired amount of sol at 37 C. is added to the serum, previously warmed to 37 C., and thoroughly shaken for 1 minute; after which the mixture is placed at once in cracked ice for 1 hour; it is then transferred to the Roux water bath at 37 C., and at intervals a portion is removed, centrifugated at 3000 revolutions for 4 minutes or more, and injected.

The results obtained by this procedure are superior to those obtained by either of the other methods, but it must not be inferred that they are strictly constant. A factor which is not controllable is the quality of the serum; even more important is the variation in the resistance of the guinea-pigs.

TABLE 49

ACTION OF AGAR-SOL-GEL ON RAT SERUM
(MIXTURES ICED FOR 1 HOUR, THEN INCUBATED AND CENTRIFUGATED)

Mixture		Guinea-Pig		Serum		Result
No.	Ratio	No.	Weight	B. C. at 37 C. (hr.)	c.c. (intravenously)	
1	0.0005:1	1	220	1	1.0	Nil
		2	215	3	"	Nil
		3	215	6	"	Nil
		4	205	12	"	3'25"
3	0.005:1	5	190	¼	0.25	4'45"
4	0.025:1	6	190	"	"	3'30"
5	0.05 :1	7	185	"	"	3'45"
6	0.1 :1	8	198	"	"	3'30"
7	0.25 :1	9	205	"	"	4
8	0.5 :1	10	200	"	"	Slight
		10a	220	1	"	Very slight
9	1 :1	11	207	¼	"	Very slight
		11a	212	1	"	Slight

In Table 49 will be found the results of experiments with several sol-gel mixtures which correspond to those employed with guinea-pig and rabbit serum. For the composition of these mixtures reference should be made to Table 23. Mixture 2 was not tested with rat serum.

It will be seen that Mixtures 3 to 7 toxified the rat serum at 37 C. within 15 minutes so that 0.25 c.c. was fatal. Noteworthy is the fact that Mixture 1, after incubation for 12 hours, rendered the serum toxic in dose of 1 c.c. The ratio of dry agar to this amount of serum is 1:400,000, or $\frac{1}{400}$ of the amount employed by Bordet in his work with

guinea-pig serum. The difference becomes more striking when it is remembered that Bordet injected from 4 to 5 c.c., and that Nathan's lowest fatal dose was 3 c.c. It should be pointed out, further, that the absolute amount of agar employed to toxify 1 c.c. of serum in this mixture was 0.0025 mg., while with Mixture 3, of which the lethal dose was 0.25 c.c., the corresponding amount of agar was only 0.00625 mg. — a reductio ad absurdum of Friedberger's hypothesis that the anaphylatoxin is derived from the cleavage of the nitrogenous constituents of agar. An absolute demonstration of its untenability will be given later when it will be shown that the mere addition of distilled water to rat serum results in the production of anaphylatoxin.

With Mixtures 8 and 9 the results were apparently poor, thus giving support to the view that an excess of agar interferes with the reaction. It must be noted, however, that the large amount of agar serves to dilute the serum, and that consequently the dose of 0.25 c.c. does not contain the full amount of serum constituents. In another experiment with Mixture 8, tests with 0.25 c.c. being made at intervals of 15 minutes, good shock effects were obtained at the quarter- and half-hour tests; while the test at 1 hour caused death in 60 minutes. Similarly, another No.-9 mixture gave good shocks at ¼, 1, 1¼, and 1½ hours; without doubt 0.5 or 1 c.c. of these sera would have proved fatal, since, as shown in Table 46, the No.-8 sol-serum mixture did kill in dose of 1 c.c., while Mixture 9 in smaller dose almost proved fatal.

Mixture 6 was used in practically all the subsequent work with rat serum, and while it usually gave, in 15 minutes, a serum which was toxic in dose of 0.25 c.c., it failed to do so on several occasions. Such sera, however, were toxic when retested at the half or three-quarter hour period, tho in one instance the mixture had to be incubated for 1 hour before it killed in this dose. Similar variations must therefore be expected with the other mixtures for reasons already pointed out.

In view of the fact that rat serum can be toxified within 15 minutes so that 0.25 c.c. is a lethal dose, it will be evident that contact for a minute or two should be quite sufficient to render 2 c.c. fatally toxic. No effort was made actually to demonstrate this speed of poison-production with sol-gel mixtures. With the plain gel-serum mixtures, however, it was repeatedly found that 7½ minutes at 37 C. sufficed to toxify 0.25 c.c., while even 2½ minutes was enough to make 1 c.c. fatally toxic (Table 47). These facts bring out clearly the striking difference between the serum of the rat and that of the guinea-pig or rabbit.

APPARENT VARIATIONS IN TOXICITY

In the preceding work with trypanosomal anaphylatoxin, as well as with that obtained by the action of agar on guinea-pig and rabbit serum, it was shown that a treated serum was not uniformly toxic, but seemed to have an oscillation or periodicity. It was accordingly desirable to ascertain whether toxic rat serum exhibited the same peculiarity. Indications of such behavior had been frequently met with in working with this serum, but, in line with prevailing views, it was believed that the failure of a toxic serum to kill on a second injection, made some time after the first fatal test, was evidence of destruction of the poison. Serial injections to test this point were not made until it became possible to obtain a serum of maximal toxicity.

A preliminary series of tests was made with a No.-7 sol-gel mixture. This was iced for 1 hour, then incubated for 20 minutes at 38 C., after which it was centrifugated at 3000 revolutions for 10 minutes. The clear serum was tested at once, the balance being placed at 38 C. and tested at intervals of 15 minutes. With 0.25 c.c. as a dose, a severe shock was obtained in the immediate test and also at 15 minutes. The tests made at ½, ¾, 1, 1¼, and 1½ hours proved fatal, while that at 1¾ hour gave a near-kill. The deaths were all acute (3½ to 4½ minutes), except in the three-quarter-hour test, in which it was protracted to 36 minutes. In the dose employed this serum was quite uniformly toxic, 5 out of 8 animals dying.

For the second trial, a No.-6 mixture was prepared in the same way as the preceding. The test made at once after centrifugation showed that 0,25 c.c. was fatal. Accordingly, the subsequent tests, at intervals of 15 minutes, were made with 0.2 c.c. The results of this experiment are given in Table 50 and are shown also in Chart 8. The fatal results in this experiment, it will be seen, were not consecutive and this variation might very well be considered as indicating an oscillation in the toxicity. Of 8 tests 4 proved fatal (compare Table 17).

A third test was made with a gel-serum mixture (0.25 : 1) for the purpose of producing a less toxic serum. The test with 0.25 c.c. made at once after centrifugation gave but a slight shock; those made at ¼, ½, and 1¼ hours killed, whereas the other tests up to 2¼ hours gave but slight or moderate effects. Hence, in this experiment of 10 tests but 3 proved fatal.

Here, as elsewhere, the question arose as to whether the variations were due to changes in the toxicity, or to unequal susceptibility on the part of the guinea-pigs. To assume the latter would be the easiest way to account for the irregularities, but, inasmuch as the matter was one of considerable importance, it was necessary to present experimental proof. This was done with rabbit serum, and the results have already been presented in Table 45. In view of those results it follows that the variations noted are to be interpreted as expressive of the indi-

vidual resistance of the guinea-pigs. The sera used in the first two experiments, in dose of 0.5 c.c., would probably have been uniformly fatal. It is to be noted that in these experiments the poison persisted for the duration of the experiment, nearly 2 hours.

TABLE 50

APPARENT VARIATION IN TOXICITY OF RAT SERUM TREATED WITH AGAR
(SOL-GEL MIXTURE 6, RATIO 0.1:1)

Guinea-Pig		Serum		Result
No.	Weight	A. C.* at 37 C. (hr.)	c.c. (intravenously)	
1	190	0	0.25	3′55″
2	206	¼	0.20	Fair shock
3	170	½	"	3′50″
4	171	¾	"	Moderate
5	173	1	"	Fair
6	182	1¼	"	3′10″
7	170	1½	"	Severe
8	173	1¾	"	4′

* A. C. = after centrifugation.

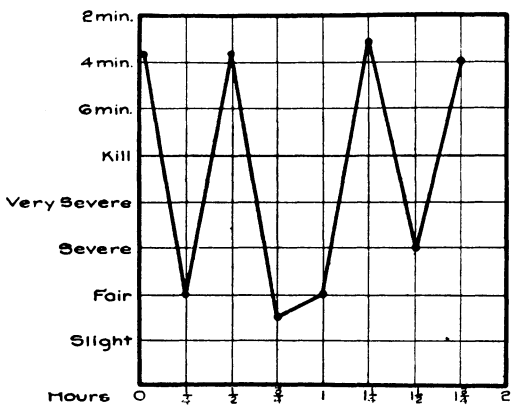

Chart 8. Apparent variation in toxicity of rat serum treated with agar (Table 50).

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

Serial Dilutions.—The observation that mixtures of agar and serum which had not been incubated, even tho they had been iced for an hour, showed no immediate toxicity, suggested that possibly adsorption of an antagonistic body might occur at 0 C., thereby setting free the ferment, which then would require only a suitable temperature to act. Were this the case, high dilutions of such treated and centrifugated serum with normal serum should toxify readily. Acting on this theory, a large number of experiments were made with iced mixtures, having

for their purpose the demonstration of the presence of a ferment (anaphylase). The very first experiment of this kind gave a most surprising result: even an E dilution, representing $\frac{1}{256}$ of the original treated serum, rendered 1 c.c. toxic after an incubation of $2\frac{1}{2}$ hours. Two of these experiments in tabulated form are sufficient to illustrate the results obtained.

The method of procedure was essentially the same as that employed in the corresponding tests with guinea-pig serum (Table 30). In the first experiment, the results of which are given in Table 51, 1.5 c.c. of agar gel were added to 6 c.c. of a pooled rat serum, both being at room temperature. The mixture was at once placed in ice and thoroughly shaken for 1 minute. It was then kept at 0 C. for 1 hour, after which it was transferred to iced tubes and centrifugated at 3000 revolutions for 6 minutes. A portion of this serum, designated as A, was tested 9 minutes later (No. 1). Another portion was placed at 37 C. and tested after 1 hour (Nos. 2 and 3). Another portion was used to make the dilution series. For the latter, 1 c.c. of A was added to 3 c.c. of the pooled serum, giving a 1:4 dilution, or B. Diluting 1 c.c. of B in like manner gave the 1:16 dilution C, and this process was repeated yielding the 1:64 dilution D, and then the 1:256 dilution E. For a control, another portion of A was placed at 60 C. for one-half hour, to destroy any ferment that might be present. The control B dilution was made by adding 1 c.c. of this heated serum to 3 c.c. of the pooled serum. These dilutions, in small Erlenmeyer flasks, were placed in a Roux water bath at 37 C., and tested at the times indicated. The same pooled serum was employed for all these tests, and it is hardly necessary to add that strictly sterile conditions obtained.

For the experiment given in Table 52, 1 c.c. of gel and 4 c.c. of rat serum, both previously kept at 0 C. for 1 hour, were mixed and thoroughly shaken for 1 minute, after which the mixture was placed in ice for 1 hour. It was then transferred to iced tubes and centrifugated at 3000 revolutions for 4 minutes. The supernatant serum, designated as A, was then used to make dilutions B, C, D, and E, the same procedure being followed as given in the foregoing.

For a control, 4 c.c. of the same pooled normal serum were heated at 60 C. for 1 hour, to destroy any ferment that might be present. This inactivated serum, cooled to 0 C., was treated with agar gel at the same time as the foregoing, the two tests running parallel. The control A thus obtained was then used to make the control dilutions (into normal serum) B, C, D, and E, which were then incubated and tested at the same time as the former. For all the tests in this experiment a single pooled serum was employed.

The results presented in Tables 51 and 52 are of interest inasmuch as, at first sight, they would tend to support the ferment theory. It will be seen that in both experiments, all 4 dilutions became toxic after a relatively short incubation. The B dilutions of both sets, it is to be noted, developed the lethal dose of 0.25 c.c.

The correct interpretation of these, and many similar experiments, centers about the inducing action of minute amounts of agar for, it is to be assumed, some agar is left in the serum after centrifugation at

3000 revolutions for 4 or 6 minutes, as was done in the foregoing. Particles of agar which have swollen by imbibition, may at 0 C. possess a density approximately that of serum, and consequently such floaters

TABLE 51

I. APPARENT FERMENT ACTION
(PROGRESSIVE DILUTION OF TREATED RAT SERUM WITH NORMAL SERUM)

Series	Guinea-Pig		Serum			Result
	No.	Weight	Dilution	Hours at 37 C.	c.c. (intravenously)	
A	1	187		0	1.0	3′20″
	2	185		1	0.5	2′45″
	3	175		1	0.25	Slight
B	4	198	1:4	1¼	1.0	3′
	5	170	1:4	1¼	0.5	3′10″
	6	185	1:4	1½	0.25	2′20″
C	7	170	1:16	1¾	1.0	3′20″
	8	170	1:16	1¾	0.5	Very severe
D	9	172	1:64	2	1.0	4′5″
	10	205	1:64	2	0.5	Slight
E	11	170	1:256	2½	1.0	3′
	12	190	1:256	2½	0.5	Very slight
Control B	13	240	1:4	1¼	1.0	3′
	14	182	1:4	1¼	0.5	Very severe

TABLE 52

II. APPARENT FERMENT ACTION
(PROGRESSIVE DILUTION OF TREATED RAT SERUM WITH NORMAL SERUM)

Series	Guinea-Pig		Serum			Result
	No.	Weight	Dilution	Hours at 37 C.	c.c. (intravenously)	
B	1	177	1:4	2	0.5	2′55″
	2	170	1:4	2	0.25	4′20″
C	3	180	1:16	2¼	0.5	4′15″
D	4	201	1:64	2½	1.0	5′
E	5	203	1:256	2½	1.0	2′40″
	6	200	1:256	2¾	0.5	3′20″
Control B	7	211	1:4	2	1.0	3′35″
Control C	8	205	1:16	2¼	1.0	5′25″
Control D	9	175	1:64	2½	1.0	3′50″
Control E	10	208	1:256	2¾	1.0	Slight

will resist the centrifugal force. This is especially true of the inactivated serum, which may retain in suspension all of the agar. This is what actually occurred in the control test in the experiment given in

Table 52. The serum which was inactivated at 60 C. for 1 hour, then treated with agar as has been indicated, gave no visible deposit after centrifugation for 4 minutes. It was consequently centrifugated again for a like period, but even then scarcely any agar was thrown down. Similar mixtures of gel and inactivated serum, when centrifugated at 8000 revolutions yielded an agar deposit, and the supernatant fluid in dilution C gave a severe shock after incubation for 2¼ hours. Dilutions D and E showed no effect. The control dilutions B, C, D, and E were made with this inactivated serum, which contained, therefore, practically all of the original agar. It will be observed from the table that the control dilutions B, C, and D (Nos. 7 to 9) became toxic.

If it is assumed that the hypothetical ferment was destroyed by heating to 60 C. for 1 hour, it follows that the effects produced by these dilutions, which were made into normal serum, were due to the transferred agar. And, since the ratio of agar to serum in the original mixture was 1:800, it follows that the ratios in the control dilutions B, C, D, and E were 1:3200; 1:12800; 1:51200; and 1:204,800, respectively.

The necessity of bearing in mind the action of minute amounts of agar will be fully realized when reference is made to the work with sol-gel mixtures (Table 49). It will be seen there that Mixture 1, having an agar-serum ratio of 1:400,000, toxified 1 c.c. of the serum, the test being made after incubation for 12 hours; while Mixture 3, the ratio in which is 1:40000, after only 15 minutes' incubation gave a lethal dose of 0.25 c.c. In view of these facts it is evident that the action of the residual agar must not be overlooked. The incompleteness of the precipitation of the agar at 3000 revolutions is demonstrable by resubmitting the serum to a higher speed, as has been pointed out in Part II. The effects of such treatment on the inducing power will be presently discussed.

Similarly, the control B dilution of Table 51, made with a portion of Serum A which had been heated at 60 C. for half an hour, became toxic, tho the amount of agar present in this instance was much less than in those mentioned above, since the inactivation took place after centrifugation for 6 minutes at 3000 revolutions. Unless it be assumed that the ferment is not destroyed by heating at 60 C., it follows that the toxicity is due either to the action of the residual agar or to that of the heated serum.

Effect of Centrifugation.—Besides the controls with heated sera,

referred to, direct attempts were made to determine the action of the residual agar in mixtures which had been centrifugated for different lengths of time, or at varying speed. One of these experiments, reproduced in Table 53, is reasonable evidence that the inducing power of a treated serum is due to the retained agar.

In this, as in the preceding experiment, the mixture of 1.5 c.c. gel and 6 c.c. of rat serum, both previously kept at 0 C. for 1 hour, was vigorously shaken for 1 minute, then placed in cracked ice for 1 hour. It was then transferred to iced tubes and centrifugated at 3000 revolutions. At the end of 2 minutes, the machine was stopped and a portion of the supernatant fluid was used to make the first series of dilutions, B, C, D, and E (Exper. 1). At this time the agar was incompletely thrown, as shown by the small deposit and by the presence of floaters; consequently this series should have become readily toxified, and such actually took place.

After the removal of the portion needed for this series of dilutions, the centrifugation of the remainder was continued for 18 minutes more, making a total of 20 minutes. As the otherwise clear supernatant serum showed about 8 large floaters, it was again centrifugated for 1 minute but without throwing the latter; a further swing for 2 minutes likewise failed. The serum with the floaters was then decanted and again centrifugated for 2 minutes with no better result. In order to avoid further delay, the remaining floaters were fished out with a platinum wire, and the clear serum was then used for the second series of dilutions (Exper. 2). It will be noted that the serum for this second series was centrifugated for 25 minutes, whereas that for the first was given but 2 minutes. The same pooled serum was used for both series of tests.

As might well be expected, Table 53 shows that the toxifying power of a treated serum is greatly reduced by an increased centrifugation. The fact that visible floaters persisted in such serum even after centrifugation for 25 minutes, makes it probable that finer particles of agar were still present, and that the comparatively slight effect obtained in the second series was due to this residuum.

As further evidence of the effect of centrifugation, it may be mentioned that several gel-serum mixtures thus treated, were centrifugated at 3000 revolutions and portions of the supernatant fluid removed at 2, 6, 12, and 24 minutes; these were then incubated at 37 C. for 3 hours and tested, the dose being 0.5 c.c.. In one trial, the first portion was fatal, while the others showed progressively less and less effect; in another trial the first and second portions produced severe shocks, while the third and fourth gave no effect. This gradation in effects is clearly due to the decrease in the amount of residual agar by the continued centrifugation.

Of the many other efforts to determine the point at issue, perhaps a few will serve as confirmatory of the preceding. The comparative effect of centrifugation at different rates of speed will be seen in the 3 experiments given in Table 54. For Exper. 1, a mixture of 1.5 c.c. of gel and 6 c.c. of distilled water, both previously kept at 0 C., was shaken as usual, then iced

for 1 hour, after which it was centrifugated at 3000 revolutions for 4 minutes. The clear supernatant water was then used to make dilutions B, C, and D.

For Exper. 2, an exactly similar mixture was made, and, after icing for 1 hour, it was centrifugated at 8000 revolutions for 20 minutes. The clear supernatant water was then used to make the dilutions B, C, and D of that series.

It was expected that these two series of tests would show the effect of any residual agar left in suspension in distilled water, after centrifugation at 3000 and 8000 revolutions, respectively; that in the case of the latter the amount of retained agar would be less with corre-

TABLE 53

COMPARATIVE EFFECT OF CENTRIFUGATION FOR 2 AND 25 MIN. AT 3000 R.P.M. (APPLIED TO A GEL-SERUM MIXTURE, RATIO 0.25:1, ICED 1 HR.)

Series	Guinea-Pig		Serum			Result
	Hr.	Weight	Dilution	Hours at 37 C.	c.c. (intravenously)	
			Exper. 1*			
B	1	205	1:4	2½	1.0	3′40″
C	2	180	1:16	"	"	3′50″
D	3	204	1:64	2¾	"	2′55″
E	4	207	1:256	"	"	Severe
	4a	200	1:256	9	"	3′″45
			Exper. 2*			
B	5	205	1:4	3¼	1.0	4′15″
C	6	205	1:16	"	"	Very slight
	6a	200	1:16	9	"	Severe
D	7	176	1:64	3¼	"	Slight
	7a	200	1:64	9	"	Very slight
E	8	202	1:256	3¼	"	Very slight
	8a	200	1:256	9	"	Nil

* The dilutions of Exper. 1 were made after centrifugation for 2 minutes; those of Exper. 2 after centrifugation of the same mixture for 25 minutes.

sponding decrease in the inducing power. When the results of Exper. 2 are compared with those of Exper. 1, this is found to be the case. The very slight effects obtained with dilutions B, C, and D of Exper. 2 are really no more than would have been obtained with a like dose of normal serum incubated for the same length of time. Consequently, Exper. 2 is to be considered as showing that at 8000 revolutions practically all the agar is thrown out of an agar-water suspension. A similar suspension centrifugated at 3000 revolutions for 4 minutes is

not freed of its agar and hence the results seen in Exper. 1. Incidentally, a comparison of the latter results with those of Table 52, where an agar-serum mixture is shown to have been centrifugated at like speed for the same length of time, shows that the denser serum probably holds up more of the agar than does the water. This is even more certain when Expers. 2 and 3 of Table 54 are compared.

For Exper. 3 a gel-serum mixture was prepared and treated in exactly the same way as for the experiment recorded in Table 52, except that it was centrifugated at 8000 revolutions for 20 minutes. A comparison of the results in Expers. 2 and 3, both mixtures having been centrifugated at the same high speed and for the same length of time, shows that the serum mixture possesses some inducing power which is not present in the agar-water mixture; in other words, the agar is not removed as completely from the serum as it is from the water suspension. A further comparison of the results obtained in Exper. 3 with those of Table 52, both being from serum mixtures but mixtures subjected to different rates of centrifugation, shows at once that the high speed enormously reduces the inducing power of a treated serum. It is reasonable to believe that centrifugation at 8000 revolutions for an hour would still further decrease this toxifying power.

It is not to be assumed that the results obtained after centrifugation at 8000 revolutions for 20 minutes (Table 54) are always equally favorable. It has occurred in similar experiments that the B and C dilutions did kill in a 1-c.c. dose, after incubation for 2¼ hours. Indeed, in one such experiment the B and C dilutions killed in dose of 0.5 c.c. In another experiment in which the centrifugation lasted but 15 minutes, even the dilution D killed after being incubated for 8 hours. Results of this kind, occurring after long incubation, must be regarded with suspicion owing to the liability of normal rat serum to become toxic through mere incubation.

With recognition of the evident fact that centrifugation does reduce the toxifying power of a treated serum, it can be assumed that this is due to the removal not merely of agar, but rather of a suspensoid form of the ferment. It is possible to conceive that the finely divided agar adsorbs the ferment and that the latter while in this state exerts its characteristic action. If such were the case, obviously, centrifugation would remove the ferment-laden agar, thereby lessening the toxifying power of the serum. It was necessary, therefore, to test this possibility by direct experimentation; and first of all, it had to be shown that the

mere process of centrifugation did not of itself destroy the activity of such suspended ferment.

When a gel-serum mixture, after icing for 1 hour, is centrifugated at 3000 revolutions for 6 minutes, and the resulting apparently clear serum is then recentrifugated at 8000 revolutions for 20 minutes, it

TABLE 54

COMPARATIVE EFFECT OF CENTRIFUGATION AT 3000 AND 8000 R.P.M.

Series	Guinea-Pig		Serum			Result
	Hr	Weight	Dilution	Hours at 37 C.	c.c. (intravenously)	
			Exper. 1*			
B	1	225	1:4	3	1	Moderate
	1a	190	1:4	3½	"	5'10"
C	2	212	1:16	3¼	"	4'25"
D	3	210	1:64	3¼	"	Slight
	3a	177	1:64	9	"	3'40"
			Exper. 2*			
B	4	171	1:4	3	1	Nil
	4a	208	1:4	10½	"	Very slight
C	5	202	1:16	3¼	"	" "
	5a	202	1:16	10½	"	" "
D	6	171	1:64	3¼	"	" "
	6a	213	1:64	10½	"	" ".
			Exper. 3†			
B	7	177	1:4	3	1	Slight
	7a	180	1:4	8½	"	2'25"
C	8	198	1:16	3	"	Very slight
	8a	230	1:16	8½	"	Very severe
D	9	185	1:64	3¼	"	Very slight
	9a	178	1:64	8¾	"	Very slight
E	10	190	1:256	3¼	"	Very slight

* In Exper. 1 a gel-water mixture was centrifugated at 3000 r.p.m. for 4 minutes and dilutions then were made. In Exper. 2 a liks mixture was swung at 8000 r.p.m. for 20 minutes.

† In Exper. 3 a gel-serum was given 8000 r.p.m. for 20 minutes.

yields a slight deposit or film on the glass. In one experiment, the deposit and the supernatant serum were stirred up and again centrifugated at the high speed. The deposit and the serum having again been thoroughly stirred up, this mixture was used to make Dilutions B and C with normal serum. On incubation for 3½ hours, these dilu-

tions caused acute death in 0.5-c.c. dose, and behaved in that respect exactly the same as the corresponding dilutions made with a serum obtained after centrifugation at slow speed. This test, therefore, served to show that high-speed centrifugation did not destroy the inducing power of the residual agar, or of the supposed ferment, but that its action consisted merely in removing the suspended matter from the serum.

Heating of the Mixture to 60 C. before Incubation.—The fact that the deposit thrown down by centrifugation, on subsequent mixture with normal serum, gives rise to poison may mean nothing more than agar action, and yet to prove that such is the case is not easy. All attempts to do so were made on the assumption that the supposed ferment was destroyed by heating at 56 C. or 60 C., and this may not be justifiable. The inactivation of a normal serum by heat does effect a marked change in the serum, since it is no longer readily toxifiable with agar or with a treated serum. This is true even when the serum is heated for a short time at 50 C., as has been shown with rabbit serum (Part III). Further evidence of this will be supplied later.

It has been shown in Table 44 that an agar serum is apparently toxified at 50 C. A similar experiment at 60 C. with rat serum may be cited at this point. A gel-serum mixture after thorough shaking for 1 minute was divided into 2 portions. The first was placed at 37 C. for 15 minutes. When tested it was found to be fatal in a dose of 0.25 c.c. The second portion was placed at 60 C. for 1 hour, after which it was also incubated at 37 C. for 15 minutes. When tested, it gave a severe shock in dose of 1 c.c. The obvious conclusion was that some poison was made, but it would be wrong to assume that this occurred either at 60 C. or during the subsequent incubation. It is more likely, and such eventually was shown to be the case, that the poison was formed before the temperature rose over 50 C. in the first heating. Hence results of this kind cannot be used to support or invalidate the ferment conception.

The one conclusion which can be drawn from such experiments is that the poison is not destroyed at the temperature employed. The heating of a toxic serum at 60 C. for 1 hour does not alter its action. Incidentally, it may be added that in one experiment a toxic serum was kept at 56 C. and when tested at 3, 6, and 24 hours was found to be fatal in dose of 1 c.c. Prolonged exposure at this temperature seems, therefore, to have very little effect on the poison.

The rat anaphylatoxin is destroyed, however, at higher temperatures as will be seen from the following experiment. A toxic serum having the lethal dose of 0.25 c.c. was dialyzed in a collodium sac at 50 C. for 15 minutes; retested it was found to kill in dose of 0.5 c.c. serum equivalent, but not in 0.25 c.c. It was then divided into 2 portions: one was placed at 70 C. for half an hour, after which it was tested and found to be fatal in dose of 1 c.c. serum equivalent, but not in 0.5 c.c.; the other portion was heated in a water bath at 100 C. for 5 minutes, and when tested it was found to be without effect in dose of 2 c.c. serum equivalent. The higher the temperature, the more rapidly is the poison destroyed.

Action of Treated Serum on Heated Normal Serum.—In view of the fact that agar does not toxify an inactivated serum very readily, it is to be expected that a treated serum would have still less action unless the residual agar had acquired a marked activity. The following experiments will show that there is no reason to believe that this does occur.

A gel-serum mixture, after icing for 1 hour, was centrifugated at 3000 revolutions for 6 minutes; a portion of this treated serum (1 c.c.) was added to 3 c.c. of normal serum thus giving Dilution B; a like portion was added to 3 c.c. of the same pooled serum but inactivated at 60 C. for 1 hour, giving the dilution B′. Of these mixtures, after incubation at 37 C. for 1 hour, the former killed in dose of 1 c.c., while the latter showed no effect even after incubation for 2 hours.

In a similar experiment, in which the mixture, however, was centrifugated for only 2 minutes, the B and C dilutions of the treated serum with normal serum were fatal, even in dose of 0.5 c.c., after incubation for 2¼ hours; the corresponding dilutions with inactivated serum (60 C. for 1 hour) gave very severe shocks in the same time, thus showing that such serum can be toxified to some extent.

In another experiment, in which the dilutions B and C were made with a serum which had been heated to 56 C. for only half an hour, after incubation for 3 hours, the former killed in dose of 1 c.c., while the latter dilution gave a very severe shock. Since the treated serum when incubated alone may develop a lethal dose of 0.25 c.c., it follows that the effect of a B dilution with inactivated serum may be due to the original serum which is carried over into the dilutions.

These illustrations appear to show that a treated serum, obtained by low-speed centrifugation, is able at times to toxify a serum which has been heated to 56 C. and even to 60 C.; the explanation is probably that just given. This result is not obtained when high-speed centrifugation is employed, because of the more complete removal of the residual agar.

Action of the Agar Deposit on Heated Normal Serum.—In view of these results, it should be possible to toxify an inactivated serum by the addition of the agar deposit obtained by centrifugation, and experiments made with this object in view were successful.

In these tests, the gel-serum mixture after icing for 1 hour, and without incubation, was centrifugated at 8000 revolutions for 20 minutes. After the removal of the supernatant fluid, the deposit was taken up with 5 c.c. of serum, previously heated to 56 C. for 1 hour, and this mixture was then incubated at 37 C. In 1 trial, the test made at 3 hours with 1 c.c. gave a severe shock, and that at 8 hours killed in 2 minutes 10 seconds. A like result was obtained in a second experiment of this kind, while in a third fairly severe shocks were obtained. Of 2 other experiments, after incubation for 3 hours, one mixture caused death in 3 minutes 5 seconds, while the other produced a severe shock.

These results are fair evidence that the agar deposit is able to toxify a serum inactivated at 56 C. Whether this is due entirely to the agar, or to an adsorbed ferment, was a question which it was hoped would be settled by suitable control experiments, which were made at the same time as those mentioned. Mixtures of gel and distilled water, or salt solution, in place of normal serum, were iced for 1 hour, after which they were centrifugated and the agar deposit taken up with the inactivated serum and incubated at 37 C. The injections made at the end of 3 and 8 hours were practically negative, showing that the plain agar under these conditions could not toxify the heated serum. These results at first sight would seem to prove the presence of a ferment in the agar deposit obtained from a treated serum. There are, however, serious objections. In the first place, it will be shown that occasionally plain agar will toxify an inactivated serum, which fact of itself excludes the question of an adsorbed ferment. Again, it is possible that the agar from an iced serum mixture may adsorb or imbibe the matrix of the poison, which on subsequent incubation with inactive serum becomes toxified.

Attempts to Extract Ferment from the Agar Deposit.—If the agar adsorbed a ferment in some such way as fibrin is said to take up thrombin, it should be possible to extract it by means of distilled water or salt solution. A number of attempts to do this failed to give unequivocal results.

Thus, in one experiment a sol-serum mixture after incubation for 1 hour at 37 C., was centrifugated, and the deposit was then taken up with 4 c.c. of salt solution and digested at 37 C. for 1 hour. This suspension was now centrifugated and 1 c.c. of the extract was added to 3 c.c. of normal serum, and this mixture, kept at 37 C., when tested after 3 and 6 hours gave a fair shock.

Again, a mixture of 3.5 c.c. of gel and 14 c.c. of serum, after being iced for 1 hour, was centrifugated at 8000 revolutions for 20 minutes, and the agar deposit taken up with 14 c.c. of distilled water. This suspension after being digested at 37 C. for one-half hour was centrifugated the same as before; the extract added to a like volume of normal serum was incubated at 37 C. for 3½ hours and tested. The equivalent of 1 c.c. of serum, the usual dose, killed in 8 minutes.

This result seemed to indicate that a ferment had been extracted from the agar deposit. It was possible, however, that a small amount of agar was redissolved and that this toxified the serum. In order to control this point, a mixture of agar gel and distilled water was treated in exactly the same way as the gel-serum mixture. The final distilled-water extract when added to an equal volume of serum and incubated gave no effect at the 3-hour test, but at 8 hours gave a severe shock. Clearly, some agar had dissolved, tho apparently less than in the experiment proper. While this explanation may account for the result obtained, it must be borne in mind that distilled water itself tends to toxify rat serum.

When the digestion of the agar precipitate with distilled water was carried on at 45 C., the inducing power of the extract appeared to be higher than that obtained at 37 C., and similarly, digestion for 1 hour was better than one for 15 minutes. The effect of heat on this active extract will be seen from the following:

The final extract obtained by treating an agar deposit with distilled water at 45 C. for 1 hour was divided into 2 portions. One portion was added to an equal volume of normal serum, and the mixture, at 37 C., gave in 3 hours a severe shock, and in 7½ hours killed in 3 minutes. A second portion of the aqueous extract was heated to 100 C. for 3 minutes, then cooled and added to an equal volume of serum; the resultant mixture was incubated and tested at the same time as the preceding. This serum with the heated extract failed to give any appreciable effect at the 3- and 7-hour tests. The agar deposit used for this test was obtained from a gel-serum mixture which had been iced but not incubated.

The experiment just given might be taken to point to the presence of a ferment, but such interpretation must be questioned. The speed of the reaction is not such as it ought to be were a ferment actually concerned. The presence of minute amounts of agar in the extracts appears to be the best explanation for the results obtained.

Action of Heated Treated Serum on Normal Serum. — Another series of attempts to show whether the action of the residual agar was due to the agar itself or to an adsorbed ferment consisted in inactivating the treated serum obtained after the centrifugation of the iced gel-serum mixture. By heating such serum to 60 C. or 70 C. or even to 100 C. it should be possible to destroy the hypothetical ferment. The dilutions of the inactivated serum with normal serum (B and C) on subsequent incubation and testing might throw some light on the question.

One experiment of this kind is to be found in Table 51 (Nos. 13 and 14). It will be seen that in this case the serum which had been heated at 60 C. for 1 hour was capable of toxifying the B dilution. In another exactly similar experiment no effect was obtained when the serum was tested at the end of 1 hour; retested after 28 hours at 37 C. it proved fatal, but no special significance is to be attached to this result, since normal rat serum on prolonged incubation may become toxic in dose of 1 c.c. In a third experiment, the treated serum being likewise heated at 60 C. for 1 hour, the B dilution after incubation for 2 hours had a slight effect, while the C dilution tested at the same time produced a severe shock. The experiments with B and C dilutions of treated sera which had been heated to 70 C. and to 100 C. were entirely negative. In order to avoid coagulation when inactivating at high temperatures, the treated serum was first dialyzed in a collodium sac against distilled water, at 50 C. for about 5 minutes.

While these results might be taken to indicate the destruction of a ferment, the more reasonable explanation seems to be that the state of the residual agar is altered by the higher temperatures and becomes less active as an inducing agent.

Action of Agar on Heated Normal Serum.—According to Bordet, agar does not toxify guinea-pig serum which has been heated to 56 C., and a like conclusion was reached by Nathan, who worked with agar, starch, and inulin. This is undoubtedly true for the conditions under which they worked. Since guinea-pig serum does not readily yield as low a toxic dose as rat serum, it is clearly not as well adapted for the study of this reaction.

The work with trypanosomes (Table 14), however, showed that rat serum, the inactivated, could be rendered poisonous, and this fact led to similar tests with agar. The very first attempt with agar resulted favorably.

In this test, a mixture of 1 c.c. of agar gel and 4 c.c. of rat serum, previously heated to 56 C. for half an hour, when incubated at 37 C. for 15 minutes and then centrifugated gave a clear serum which in dose of 2 c.c. caused typical shock and death in 4 minutes 30 seconds. A control experiment made at the same time with normal serum gave a lethal dose of 0.25 c.c. In 5 other tests, made at different times, with 1 c.c. as the dose, little or no effect was obtained. It has been shown that the agar deposit after centrifugation of a gel-serum mixture can toxify an inactivated serum so that 1 c.c. is fatal.

It appeared from these tests that the inactivated serum was less easily toxified than normal serum, and that the toxicity secured was but a fraction of that which can be gotten with normal serum.

In the course of the work of the following year it was realized that the behavior of serum when heated was deserving of a more extended study. It will be shown in Part IX that even a short exposure at 50 C. alters the serum, and renders it less toxifiable by addition of distilled water. This observation led to similar tests with agar, which will be considered at this point.

Inactivation at 50 C.—It has been shown in connection with Table 44 that while agar apparently is able to toxify rabbit serum at 50 C., in reality the poison is produced before this temperature is reached. In view of this fact it was desirable to ascertain the action of agar on rat serum when incubated at 45 C., it being possible that this temperature was more favorable for poison-production than that of 37 C.

For this purpose a sol-gel serum mixture (No. 7) was prepared, the sol and serum being each warmed to 45 C. (the latter for one-half hour) before mixing; it was then iced for 2 hours, after which it was placed at 45 C. and tested as indicated in Exper. 1, Table 55. It will be seen that under these conditions agar readily toxifies the serum, giving a lethal dose of 0.25 c.c. about as readily as at 37 C.

For Exper. 2, a portion of the same pooled serum, previously heated to 50 C. for half an hour, was treated with agar gel (1:4); after vigorous mixing for 1 minute, the mixture was placed at once at 45 C. and tests were made, in pairs, at 15-minute intervals, as shown in Table 55 (see Table 45).

A strict comparison of the two experiments is unfortunately impossible since in the former the sol-gel method was employed while in the latter the gel procedure was used. Nevertheless, it will be apparent that the serum which had been heated to 50 C. was less reactive than that exposed to 45 C. for a like time.

This experiment with a serum previously heated at 50 C. for half an hour was repeated on the following day, the method of procedure being exactly the same. It will be seen that altho the speed of the reaction appears to be less than usual, the results are very favorable, since even the lethal dose of 0.25 c.c. was obtained (Table 55, Exper. 8).

Inactivation at 56 C.—The results with serum inactivated at 56 C. showed an enormous decrease in capacity to produce anaphylatoxin. This will be apparent on comparison of Tables 55 and 56.

For Exper. 1 of Table 56, the serum was heated at 56 C. for half an hour; it was then treated with gel, as in the preceding test, after which the mixture was incubated at 45 C. and tested. The fact that 1 c.c. of this treated serum caused a good shock in 2 of the tests indicated that a fatal result could be expected with twice that dose. Accordingly, the experiment was repeated, tho with a different pooled serum which had been also heated to 56 C. for half an hour. This was used for Expers. 2 and 3 (Table 56), the former mixture being incubated at 45 C., the latter at 38 C. It will be seen

from the table that the treated inactivated serum, even in dose of 2 c.c., was only exceptionally toxic.

It was thought that possibly better results could be obtained by dialyzing the serum in a collodium sac at 55 C. for half an hour. In Exper. 4, made with this object in view, the volume of the serum was doubled in the course of the dialysis. The agar gel was then added to the dialyzed serum (1:4), thoroughly shaken, and the mixture placed at 38 C. The equivalent of 2 c.c.

TABLE 55

ACTION OF AGAR ON RAT SERUM WHICH HAS BEEN HEATED FOR HALF AN HOUR AT 45 C. (EXPER. 1); AND AT 50 C. (EXPERS. 2 AND 3)

Guinea-Pig		Serum		Result
No.	Weight	Hours at 45 C.	c.c. (intravenously)	
		Exper. 1*		
1	203	¼	1.0	3′ 5″
1a	202	¼	1.0	3′40″
2	186	½	0.5	3′40″
2a	185	½	0.5	2′40″
3	190	½	0.25	3′30″
3a	190	½	0.25	Very near-kill
		Exper. 2*		
1	204	¼	1.0	Moderate
1a	207	¼	1.0	3′40″
2	190	½	1.0	Moderate
2a	190	½	1.0	4′30″
3	195	¾	1.0	4′15″
3a	190	¾	1.0	4′
4	196	1	0.5	Slight
4a	196	1	0.5	Slight
		Exper. 3*		
1	195	¼	1.0	4′30″
1a	193	¼	1.0	2′50″
2	188	½	0.5	Good
2a	187	½	0.5	Good
3	202	¾	0.5	4′45″
3a	203	¾	0.5	2′40″
4	178	1	0.25	3′45″
4a	175	1	0.25	Severe

* Duplicate inoculations were made at each interval as shown in table.

of serum was used as the dose. It will be seen that in this experiment the toxic effects were a trifle more marked than in the others.

This result led to further experiments with serum dialyzed at 50 C. for 15 minutes, then heated in a test tube at 56 C. for half an hour. The serum thus inactivated was then treated either by the gel or the sol-gel method. Of 3 such experiments, 2 gave little or no evidence of poison-production, while 1 developed a very severe shock after incubation for 30 minutes.

It will be seen from the foregoing, that while it is possible at times to toxify a rat serum which has been inactivated by heating at 56 C., more often the results are negative. Obviously, a larger dose than 2 c.c. might give more pronounced effects. As the matter stands, however, it is clear that the change inaugurated by heating at 50 C., becomes more complete by exposure to 56 C.

TABLE 56

ACTION OF AGAR ON RAT SERUM WHICH HAD BEEN HEATED FOR HALF AN HOUR AT 56 C.

Guinea-Pig		Serum		Result
No.	Weight	Hours at 45 C. or 38 C.	c.c. (intravenously)	
		Exper. 1*		
1	185	¼	1	Moderate
1a	187	¼	"	Severe
2	202	½	"	Slight
2a	205	½	"	Good
3	185	¾	"	Very slight
		Exper. 2*		
1	205	¼	2	Very slight
1a	203	¼	"	Moderate
2	198	½	"	Moderate
2a	202	½	"	Slight
		Exper. 3*		
1	197	¼	2	3'10"
1a	197	¼	"	Slight
2	190	½	"	Slight
4a	191	½	"	Very slight
		Exper. 4*		
1	188	¼	2	Severe
1a	188	¼	"	2'30"
2	185	½	"	Good
2a	186	½	"	Moderate

* Tests made in duplicate. Mixtures for Expers. 1 and 2 were kept at 45 C.; those for Expers. 8 and 9 were kept at 38 C.

The interpretation of these results is of fundamental importance. It may be assumed that the heat of inactivation destroys the ferment, but it is more likely that it causes a change in the matrix. The indication is that the matrix, probably but a small part of the total protein of the serum, like fibrinogen is very labile. It may be imagined that

the action of heat consists first in a dispersion followed by an intramolecular change analogous to that which occurs in heat coagulation. Once denatured by heat, the capacity of the matrix to produce poison is lost.

Toxicity of the Agar Deposit.—Rat serum on incubation with agar yields a precipitate which is more abundant than that obtained with guinea-pig or rabbit serum, and on centrifugation this precipitate is thrown down along with the agar. Since an adsorbing surface such as that presented by agar might possibly take up anaphylatoxin from the serum, many efforts were made to detoxify a serum by repeated treatments with agar gel. The results seemed to indicate that the poison could be removed in this way.

If an adsorption of some of the poison does take place, then it should be possible to obtain indications of its presence by digestion of the agar precipitate with distilled water. Several attempts were made with this object in view.

In one experiment a sol-gel mixture, consisting of 2.5 c.c. of sol and 10 c.c. of rat serum, after icing for 1 hour, was incubated at 37 C. for 20 minutes and then centrifugated. The supernatant serum in dose of 0.25 c.c. gave a good shock when tested at once and after 10 minutes. The agar deposit was rubbed up with 10 c.c. of distilled water and placed at 45 C. for 1 hour, after which it was centrifugated for 5 minutes and the clear aqueous extract injected. The first test with 5 c.c. caused typical acute death in 2 minutes 40 seconds in a guinea-pig weighing 206 gm.; and a second test with 2.5 c.c. was fatal in 30 minutes to a guinea-pig weighing 170 gm.

In another experiment a similar result was obtained. The sol-gel mixture in this case consisted of 2.2 c.c. of sol and 22 c.c. of serum; after being iced for 1 hour it was placed at 38 C. for 15 minutes and then centrifugated. The supernatant serum was fatal in dose of 0.25 c.c. The agar deposit from this serum was taken up with 22 c.c. of distilled water and digested for 1 hour at 38 C., after which the mixture was centrifugated and the aqueous extract tested. Injected in dose of 5 c.c. into a guinea-pig weighing 200 gm. it caused typical shock and death in 3 minutes 10 seconds.

The question arose as to whether the toxicity of the extract was due to the re-solution of an adsorbed or occluded poison, or whether it was to be correlated with the toxicity of organ extracts. The former view was supported by the following experiment in which the serum precipitate was excluded.

A clear toxic rat serum, the lethal dose of which was 0.25 c.c., was mixed with an equal volume of gel (3.75 c.c.) and set aside at 0 C. for 21 hours. The mixture was then centrifugated; 1 c.c. of the clear serum when injected into a guinea-pig produced but slight effect. Evidently, the toxicity had greatly decreased, possibly as the result of adsorption. The agar deposit was then

digested with 15 c.c. of salt solution at 50 C. for 45 minutes and centrifugated. The injection of 10 c.c. of this extract into a guinea-pig weighing 230 gm. caused death in 2 minutes 40 seconds, while 5 c.c. produced but slight effect. The agar extracted a second time with 15 c.c. of salt solution, at 50 C. for 1 hour, gave an extract which in dose of 10 c.c. had practically no effect.

It is evident, therefore, that the agar may adsorb or what is more likely, occlude a certain amount of the serum constituents including the poison. This fact serves to explain why, at times, apparently large doses of agar fail to toxify a serum.

SUMMARY

Rat serum can be toxified with agar in 7½ minutes so that 0.25 c.c. will cause acute fatal shock; after incubation for only 2½ minutes, 1 c.c. may be fatal. The reaction concerned in the production of anaphylatoxin is one of great speed. Agar and trypanosomes can work at the same speed since they can toxify 1 c.c. in 2½ minutes.

The individual sera show some variation in the ease with which they can be toxified. This is not due to the age of the serum or to the presence of lipoids. Normal rat serum, without any addition, on long incubation may become toxic so that 1 c.c. will be fatal.

The physical state of the agar is an important factor in the production of the poison. Its inducing power is not affected by sterilization at 140 C.

The sol at 37 C. is inferior to the solid or semisolid agar, and the best results are obtainable by the sol-gel method.

A mixture (No. 1) containing 0.0005 c.c. of agar per cubic centimeter of serum can be toxified so that 1 c.c. will be fatal. This amount of agar solution contains 0.0025 mg. of dry agar — a quantity so small as to exclude it as the source of the poison, and a reductio ad absurdum of Friedberger's theory of anaphylatoxin-production. The ratio of dry agar to serum in this mixture is 1 : 400,000.

The most convenient mixture, probably, is one containing 0.1 to 0.05 c.c. agar per cubic centimeter of serum. The reaction is not inhibited in a mixture consisting of equal parts of agar and serum.

Some incubation of the agar serum at or near 37 C. is necessary to the production of the poison. Incubation at 45 C. yields about as active a poison as at 37 C.

The poison is not destroyed when kept at 56 C. for 24 hours; it is partially destroyed in half an hour at 70 C., and apparently completely in 5 minutes at 100 C.

The apparent variation in the toxicity of a given treated serum is due to the varying resistance of the recipients.

Attempts at demonstrating the presence of a causal ferment, by progressive dilution, gave results which are shown to be due to the residual agar. No positive evidence that agar adsorbed a ferment could be established.

Serum which has been heated to 56 C., or even to 60 C., can be toxified by a treated serum, or by the agar deposit, or even by plain agar, but not as readily as unheated normal serum. The serum is weakened by heating to 50 C., but not by heating to 45 C. The facts indicate that the matrix of the poison is very labile.

The agar deposit may hold, probably by occlusion, one or more fatal doses of the poison.